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Hypoxia stabilizes GAS6/AXl signaling in metastatic prostate cancer

Anjali Mishra[1,2], **Jingcheng Wang**[1], **Yusuke Shiozawa**[1], **Samantha McGee**[1], **Jinkoo Kim**[1], **Younghun Jung**[1], **Jeena Joseph**[1], **Janice E. Berry**[1], **Aaron Havens**[1], **Kenneth J. Pienta**[3,*], and **Russell S. Taichman**[1,*]

[1]Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, Michigan, USA

[2]Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, Ann Arbor, Michigan, USA

[3]Departments of Urology and Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan, USA

Abstract

The receptor tyrosine kinase Axl is over-expressed in a variety of cancers and is known to play a role in proliferation and invasion. Previous data from our lab indicates that Axl and its ligand GAS6 may play a role in establishing metastatic dormancy in the bone marrow microenvironment. In the current study, we found that Axl is highly expressed in metastatic prostate cancer (PCa) cell lines PC3 and DU145 and has negligible levels of expression in a non-metastatic cancer cell line LNCaP. Knockdown of Axl in PC3 and DU145 cells resulted in decreased expression of several mesenchymal markers including Snail, Slug, and N-cadherin, and enhanced expression of the epithelial marker E-cadherin, suggesting that Axl is involved in the epithelial to mesenchymal transition in PCa cells. The Axl-knockdown PC3 and DU145 cells also displayed decreased *in vitro* migration and invasion. Interestingly, when PC3 and DU145 cells were treated with GAS6, Axl protein levels were down-regulated. Moreover, CoCl2, a hypoxia mimicking agent, prevented GAS6 mediated down-regulation of Axl in these cell lines. Immunochemical staining of human PCa tissue microarrays demonstrated that Axl, GAS6 and Hif1-α (indicator of hypoxia) were all co-expressed in PCa and in bone metastases, compared to normal tissues. Together, our studies indicate that Axl plays a crucial role in PCa metastasis, and that GAS6 regulates the expression of Axl. Importantly, in a hypoxic tumor microenvironment Axl expression maintained leading to enhanced signaling.

Keywords

Bone Metastasis; prostate cancer; EMT; Hypoxia; tumor microenvironment; GAS6; Axl

Introduction

Cancer metastasis remains one of the most challenging clinical problems. In the United States alone, more than 350,000 people die each year due to skeletal complications associated with bone metastasis common in many cancers such as prostate and breast cancer (1). The 5 year survival rate of primary prostate cancer (PCa) is nearly 100%, however after

Correspondence: Russell S. Taichman D.M.D., D.M.Sc. Periodontics & Oral Medicine, University of Michigan School of Dentistry, 1011 North University Ave., Ann Arbor, MI 48109-1078, USA, rtaich@umich.edu.
*Both authors contributed equally to the work.

the detection of bone metastasis the 5 year survival rate declines to 33% (2). In order for cancer cells to metastasize they must at first detach from the primary tumor, invade the surrounding extracellular matrix, and cross the endothelium to enter the blood stream (3). Cancer cells must then move across the endothelium and invade the extracellular matrix before migrating and taking up residence in distant organs such as bone. Understanding this multi-step process is therefore crucial for elucidating the molecular events that produce a lethal phenotype.

Axl is one member of a tyrosine kinase receptor family (RTK), which also includes Sky (Tyro3) and Mer. Together, Axl, Sky and Mer, or the TAM (Tyro3-Axl-Mer) family of RTKs are characterized by an extracellular domain containing two immunoglobulin-like domains and two fibronectin type 3–like domains. Axl encodes a 140kD protein and was originally identified as a transforming gene in chronic myelogenous leukemia patients (4, 5) and in chronic myeloproliferative disorders (6). Growth arrest specific-6 (GAS6), is a vitamin K-dependent ligand for Axl, Sky and Mer (7–9), so named because it was initially discovered as a protein which is over-expressed in growth arrested cells (10). Binding of GAS6 leads to Axl receptor dimerization and autophosphorylation on its tyrosine resides, which triggers a cascade of intracellular signaling (11). Several studies have indicated that Axl plays a role in tumor invasion and metastasis in a number of cancers, including breast, pancreatic, glioblastoma, hepatocellular, and renal carcinomas (12–18). The expression level of Axl is directly proportional to tumor grade and predicts poor patient survival rate (19–25).

At present, less is known as to the biological role played by Axl in PCa progression. Previous findings from our lab have shown that Axl is highly expressed in metastatic PCa, and suggested that the interaction of GAS6 and TAM RTKs may play a role in establishing tumor dormancy in the bone marrow microenvironment (26). In this present report, we demonstrate that Axl, which is highly expressed in metastatic PCa cells, promotes migration and invasion of PCa cells *in vitro* and plays an important role in regulating expression of genes involved in epithelial to mesenchymal transition (EMT) of cancer cells. Our studies also reveal that Axl expression levels are negatively regulated by its ligand GAS6. However, in hypoxic environments such as in a tumor or in bone metastases, down-regulation of Axl expression is prevented. Together these studies demonstrate that Axl plays a crucial role in PCa metastasis, and its expression is regulated by both GAS6 and hypoxia.

Materials and Methods

Cell culture

PC3 (CRL-1435), DU145 (HTB-81), and LNCaP (CRL-1740) PCa cell lines were obtained from the American Type Culture Collection (Rockville, MD). All PCa cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen) and 1% (v/v) penicillin-streptomycin (Invitrogen) and maintained at 37°C, 5% CO_2, and 100% humidity.

Antibodies and Reagents

Anti-Axl antibody (Cat. 4939), anti-Slug antibody (Cat. 9585), anti-Snail antibody (Cat. 3879), anti-E-cadherin antibody (Cat. 3195), anti-N-cadherin antibody (Cat. 4061), and anti-β-actin antibody (Cat. 4970) were purchased from Cell Signaling Technology (Danvers, MA). For immunostaining, anti-human GAS6 polyclonal antibody (Cat. AF885, R&D Systems, Minneapolis, MN) was detected using a Zenon Alexa Fluor 555 goat IgG labeling kit (Invitrogen). Staining with an Axl antibody (Cat. AF154, R&D Systems) and mouse anti human Hif-1α (Cat. 610958, BD Biosciences) were detected using a Zenon Alexa Fluor 488

mouse IgG1 labeling kit (Invitrogen). Human recombinant GAS6 was purchased from R&D Systems. CoCl2 (Cat. C8861) was purchased from Sigma-Aldrich (St. Louis, MO).

Axl Knockdown by Lentiviruses

GIPZ Lentiviral shRNAmir vectors containing either Axl shRNA or non-silencing (scrambled) shRNA were purchased from Open Biosystems (Huntsville, AL). Lentiviruses were constructed at the Vector Core in University of Michigan. Stable Axl knockdowns in DU145 and PC3 cells were generated by lentiviral infection followed by 1 week of selection with 1μg/ml of puromycin. Viral infection efficiency was assessed by determining the percent of GFP positive cells. Axl knockdown in these cells was confirmed by both realtime PCR and western blot analysis.

RNA extraction and Realtime PCR analysis

Realtime PCR was carried out using standard techniques. Briefly, total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA), and first-strand complementary DNA was synthesized in a 20-μl reaction volume using 0.4 μg of total RNA. Reverse transcription products were analyzed by realtime PCR in TaqMan Gene Expression Assays of several target genes including GAS6, Axl, Sky, Mer, Slug, Snail, N-cadherin, E-cadherin, and β-actin (Applied Biosystems, Foster City, CA). Real-time PCR analysis was performed using 15.0 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), 1.5 μl of TaqMan Gene Expression Assay, 1 μl of complementary DNA, and RNAse/DNAse-free water in a total volume of 30 μl. All sample concentrations were standardized in each reaction to exclude false-positive results. Reactions without template and/or enzyme were used as negative controls. The second-step PCR (95°C for 30 seconds and 60°C for 1 minute) was run for 40 cycles after an initial single cycle of 95°C for 15 minutes to activate the Taq polymerase. The PCR product was detected as an increase in fluorescence using an ABI PRISM 7700 sequence detection system (Applied Biosystems). RNA quantity (C_R) was normalized to the housekeeping gene β-actin control by using the formula $C_R = 2^{(40 - C_t \text{ of sample}) - (40 - C_t \text{ of control})}$. The threshold cycle ($C_t$) is the cycle at which a significant increase in fluorescence occurs.

Western blot analysis

PCa cells (1×10^6 cells/well) were cultured in six-well plates in RPMI medium. For Axl knockdown and EMT studies, whole cell lysates were made from cells grown in media containing serum. For GAS6 regulation studies PCa cells were serum starved overnight, after which they were either left untreated or treated with 100ng/ml of GAS6 for 6 hrs and overnight, or with 100 μM of cobalt chloride (CoCl2) for 6 hrs, 7 hrs, and overnight. Whole cell lysates were prepared from cells, separated on 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membranes were incubated with 5% milk for 1 hr and incubated with primary antibodies overnight at 4°C. Primary antibody was used a 1:1000 ratio with 5% dry milk. Blots were incubated with peroxidase-coupled secondary antibodies (Promega, Madison, WI) for 1h at a ratio of 1:10000, and protein expression was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). Membranes were re-probed with polyclonal anti-β-actin antibody (1:1000; Cell Signaling) to control for equal loading.

In vitro migration and invasion assays

In vitro migration and invasion assays were done using the Corning Cell migration, Chemotaxis, and Invasion Assay protocol. PC3 and DU145 cells were serum starved overnight. 2×10^5 cells were plated in the upper chamber of a 24 well trans-membrane permeable support with 8 μm pore size (Cat. 3422, Corning, Lowell, MA). For the invasion

assay only, 100 μl of basement membrane extract (BME) coating solution (Cat. 3455-096-02, Trevigen, Gaithersburg, MD) was placed in the top chamber before cell plating. GAS6 (100ng/ml) in serum free media or serum containing media were used as chemo-attractants in the bottom chamber. After 24 hrs the cells which had migrated or invaded were dissociated using a cell dissociation buffer (Cat. 3455-096-05) (Trevigen). Cells cells were then stained with Calcein AM fluorescent dye (Cat. C3100MP, Molecular Probes, Invitrogen) for 1 hr and placed in a 96 well solid black microplate (Cat. 3916, Corning). At the end of the experiments, the fluorescent intensities of bottom chambers were read by a fluorescent top plate reader at 485 nM excitation and 520 nM emission. A standard curve was made by staining known numbers of cells with Calcein AM. Fluorescent data from migration and invasion assay experiments were converted to cell numbers based on this standard curve.

Tumor tissue microarrays (TMA) and immunostaining

Human tissue microarrays were provided by the Prostate SPORE Tissue Core Laboratory, Urology Department, University of Michigan, for immunofluorescence staining. TMA sections were unmasked with pepsin solution (Lab Vision, Fremont CA) at 37°C for 15 min, and pH balanced with PBT (PBS plus 0.2% Triton X-100) at room temperature for 5 min. Sections were blocked with Image-iT FX signal enhancer (Invitrogen) for 30 min before incubating with fluorescence-labeled primary antibodies at room temperature for 2 hrs in the dark. Sections were washed with PBS, fixed with 10% formalin (Sigma-Aldrich) for 10 min, mounted with ProLong Gold antifade reagent with DAPI (Invitrogen), and covered with cover glass. Images were taken with Olympus FV-500 confocal microscope. For data analysis, Software NIS Elements BR3.2 64-bit (Nikon) was used in conjunction with 20x DIC M (0.19um/px) for all 20x images using an automated mass field measurement. For single color, GAS6 or AXL 555 mono wavelength channels were utilized, 488 and 555 wavelengths were used to quantify co-localization with Hif1α. Data is presented as percent positive of each 20x field.

Statistical analysis

All *in vitro* experiments were performed at least three times with similar results. Results from representative assays are shown. Numerical data are expressed as mean ± standard deviation. Significance of the difference between two measurements was determined by unpaired Student's *t*-test. Values of $p < 0.05$ were considered significant.

Results

Metastatic PCa cells express high levels of Axl

Our earlier study indicated that Axl was highly expressed in PCa cell lines (26). In that study, immunostaining of Axl in TMA samples revealed that Axl expression had a positive correlation with tumor grade. In order to confirm our earlier observations, real time PCR was performed to detect mRNA expression levels of the GAS6 receptors Axl, Sky and Mer in the metastatic PCa cell lines PC3 and DU145 and in a non-metastatic cell line LNCaP. As expected, Axl was highly expressed in metastatic cancer cells PC3 and DU145, whereas very low levels of Axl were detected in the non-metastatic cell line LNCaP (Figure 1A). Little or no expression of Sky and Mer was observed in all cell lines (Figure 1A). These results corroborate our earlier data and strongly indicate that Axl plays a role in metastasis in PCa cells. To further explore the biology of Axl expression in Pca cells, Axl expression was down-regulated using a lentivirus vector harboring either scrambled shRNA (sc.shRNA) or Axl shRNA. Real time PCR analysis confirmed Axl shRNA induced knockdown of Axl mRNA in PC3 and DU145 compared to controls (Figure 1B, C).

Axl regulates EMT in PCa cells

The process of EMT allows a cancer cell to undergo biochemical changes to assume a mesenchymal phenotype. This results in cancer cells which have enhanced migratory capacity, increased invasiveness, enhanced resistance to apoptosis, and which exhibit an ability to degrade the extra cellular matrix (ECM) components (27). In order to determine whether Axl plays a role in regulating the process of EMT, western blot analysis was performed to compare the protein levels of molecules involved in the process of EMT. As shown in Figure 2A, 2B, 2D PC3 and DU145 cells in which Axl expression is down-regulated expressed diminished levels of the mesenchymal markers Snail, Slug, and N-cadherin, and enhanced levels of the epithelial marker E-cadherin. While there were changes in the protein levels of these EMT markers, no changes in mRNA levels were seen following Axl knockdown in PC3 cells (Figure 2C) and DU145 (not shown), suggesting that Axl regulates EMT marker expression at the protein level.

Knockdown of Axl in PCa cells leads to inhibition of migration and invasion in vitro

In order to determine if expression of Axl plays a functional role in PCa metastasis, *in vitro* migration and invasion assays were performed. Following overnight serum starvation, migration across a Boyden chamber towards serum or GAS6 decreased significantly for PC3 and DU145 cells in which Axl was knocked down, as compared control cells (Figure 3A, C). Similarly, *in vitro* invasion assays were also performed to determine the invasion potential of Axl knockdown PC3 and DU145 cells. Similar to the migration assay, PC3 and DU145 cell invasion across a Boyden chamber was suppressed when Axl expression was reduced (Figure 3B, D). Together these *in vitro* data suggest that Axl plays a role in promoting migration and invasion of PCa cells.

Exogenous treatment with GAS6 reduces Axl protein expression in PCa cells

In order to determine whether GAS6 regulates the expression of its receptor, Axl, PC3 and DU145 cells were treated with GAS6 at different time points following overnight serum starvation. Axl protein levels in both cell lines were decreased at 6 hrs following GAS6 treatment. Moreover, Axl protein levels remained low even after overnight treatment with GAS6 (Figure 4A, B). While Axl protein levels went down upon GAS6 treatment, there were no significant changes in Axl mRNA levels (Figure 4C, D). This indicates that GAS6 regulation of Axl expression is mediated at the protein rather than the mRNA level.

Hypoxia prevents GAS6 mediated down-regulation of Axl in PCa cells

Several studies have indicated that the tumor microenvironment is hypoxic in nature (28). CoCl2, a reagent which mimics a hypoxic environment when added *in vitro* (29), was used to create a hypoxic environment for PC3 and DU145 cells in culture. Interestingly, CoCl2 treatment prevented GAS6 down-regulation of Axl protein expression in both PC3 and DU145 cells (Figure 5A, B). To verify CoCl2's role as a hypoxia mimicking agent, Hifl-α levels were analyzed in PC3 and DU145 cells after CoCl2 treatment. It was observed that Hifl-α levels increased within 6 hrs of treatment with CoCl2 treatment of both PC3 and DU145 cells (Figure 5C). Our results indicate that hypoxia stabilizes Axl protein and prevents its degradation via GAS6.

GAS6 and Axl co-localize with Hif1-α in both primary tumors and bone metastasis in human prostate TMA samples

Our *in vitro* studies indicate that GAS6 negatively regulates Axl protein levels, y et in hypoxic conditions Axl protein levels do not change upon in response to GAS6 stimulation. It was therefore critical to explore whether this *in vitro* result is also true in the hypoxic regions of tumors (as defined by Hif1-α staining) (30). Consequently, human PCa tissue

microarrays (TMA) were probed with antibodies that target GAS6 and Hifl-α or Axl and Hif1-α in order to determine their co-localization. Overall, Hif1-α levels were higher in prostate tumors tumor as compared to the normal prostate (Figures 6). It was also observed that GAS6 is highly expressed in regions positive for Hif1-α in both primary tumor and in bone metastasis (Figures 6). Similarly it was seen that Axl was expressed in regions positive for Hif1-α in both primary tumors and significantly in bone metastasis (Figures 7). Thus, both GAS6 and Axl are expressed within hypoxic regions of a tumor, and significantly in metastases. Together these results demonstrate that GAS6 negatively regulates Axl under normoxic conditions (Figure 4, 5), but not under hypoxic conditions *in vitro*, and within a hypoxic tumor Axl expression remains high (Figure 7) despite the presence of abundant GAS6 expression (Figure 6). These data suggest that the continuous GAS6-Axl signal in regions of hypoxia may contribute significantly to migration, invasion and metastasis.

Discussion

In this study we evaluated the role of Axl in PCa progression. We observed that reducing the expression of Axl in PCa cell lines significantly decreased both the *in vitro* migration and the invasion of cancer cells across a Boyden chamber towards either GAS6 or serum. Reducing Axl expression also resulted in the down-regulation of several mesenchymal markers including Snail, Slug and N-cadherin, while at the same time enhanced expression of the epithelial marker E-cadherin. Not surprisingly, the expression of Axl is negatively regulated by the presence of its ligand. However under hypoxic conditions, such as those in the the bone marrow or in a tumor setting, GAS6 does not down-regulate Axl expression, which may result in tumor progression towards an EMT state.

The physiological role of Axl and its ligand GAS6 has been extensively studied in vascular smooth muscle cells and in neurons. GAS6 stimulation of Axl plays a role in chemotaxis in these cells (31, 32). Recently, the role of Axl and GAS6 has been studied in a variety of cancers (12). Enhanced Axl expression correlates with increased lymph node metastasis and poor clinical outcomes in oral squamous cell carcinoma (19). Down-regulation of Axl in hepatocellular carcinoma attenuates proliferation and migration *in vitro* and peripheral lymph node metastasis *in vivo* (15). Axl was found to be over expressed in 70% of stage 2 pancreatic ductal adenocarcinoma (PDA) samples. GAS6 and Axl over-expression in PDA samples are associated with poor prognosis in patients with stage 2 PDA (12), and Axl expression in pancreatic cancer is significantly associated with lymph node metastasis (12). Axl knockdown prevents migration and invasion in pancreatic cancer cell lines and results in decreased amounts of activated (GTP-bound) GTPase proteins Rho and Rac, significant down-regulation in transcript levels of the EMT-associated transcription factors Slug, Snail and Twist, and significant decrease in matrix metalloproteinase MMP-9 mRNA levels (21). The presence of Axl in primary breast cancers predicts reduced overall survival. In mammary epithelial cells Axl is induced by EMT and Axl knockdown prevents the spread of highly metastatic breast carcinoma cells to lymph nodes and several major organs (17). Over-expression of Axl in lung adenocarcinomas and gliomas also predicts a less than favorable outcome (24, 25). Several strategies are being developed to inhibit GAS6 signaling in cancer (33). Using mouse models it has been shown that a small molecule inhibitor of Axl kinase, R428, significantly reduces metastasis of breast cancer and extends survival (34).

Little is known about how Axl expression is regulated in cancer cells. In breast cancer cells, the intermediate filament and mesenchymal marker vimentin positively regulates Axl expression (13). In colorectal and cervical carcinoma cell lines, myeloid zinc finger protein 1 (MZF1) binds to the Axl promoter, transactivates promoter activity, and enhances Axl-mRNA and protein expression in a dose-dependent manner (14). In Axl transformed

NIH-3T3 cells, Axl is post-translationally regulated by proteolytic-mediated degradation, and half life of Axl is around 2 hrs (4). This indicates that Axl protein is highly unstable and is rapidly degraded under certain conditions. Studies in human lens epithelial cells have indicated that GAS6 promotes down-regulation of Axl protein by inducing phosphorylation and ubiquitination of Axl and the interaction of Axl with the ubiquitin ligase c-Cbl (35).

In this paper, we used a hypoxia mimicking agent, CoCl2, to demonstrate that GAS6 secretion is not able to negatively regulate expression Axl in PC3 and DU145 cells under hypoxic conditions. Quite likely Axl protein is expression is stabilized in a hypoxic microenvironment since we observed very little alteration in Axl mRNA expression in response to GAS6 signaling. Regions of the tumor microenvironment are known to be very hypoxic (28). In a primary tumor, hypoxia induces an EMT, while in the bone hypoxia may increase the expression of transcription of factors which can further promote skeletal metastasis of the tumor (36). Hif-1α is a highly unstable protein that under normoxic conditions is degraded by proteolytic-mediated degradation. Hypoxia stabilizes Hif1-α protein by preventing its degradation (37). We observed that in primary PCa lesions and in bone metastases that Axl co-localizes with GAS6 and Hif-1α. These findings suggest that Axl expression under hypoxic conditions may no longer be regulated by the GAS6 ligand, and therefore may remain available for signaling or for the promotion of an EMT-like state which may drive progression of metastasis.

Earlier we showed that GAS6 is produced by primary human osteoblasts (26, 38). We have shown that in acute lymphoblastic leukemia cells migrate towards the bone using the GAS6/Mer axis. In PCa we have shown that GAS6 production by osteoblasts influences tumor dormancy and thereby protects tumor cells from chemotherapy-induced apoptosis. There have been other studies using melanoma, breast, pancreatic and colon cancer cell lines in which cancer cells were shown to promote their own growth by educating infiltrating leukocytes to up-regulate the production of GAS6 (39). These observations indicate that GAS6 production is high in the bone marrow microenvironment and that the presence of a tumor further promotes GAS6 production. Yet GAS6 can either be produced by osteoblasts (paracrine loop) or by the tumor cells themselves (autocrine loop). The hypoxic microenvironment in the bone may facilitate the high expression of Axl by the tumor cells in spite of high GAS6 expression. This results in increased production of both the ligand and receptor, which boosts GAS6/Axl signaling within the bone microenvironment that in turn drives tumorigenesis (Model in Figure 8). A similar phenomenon can be imagined in a primary PCa microenvironment, which is also highly hypoxic in nature.

It is well known that normal cells control the length and intensity of receptor tyrosine kinase (RTK) signaling by receptor down-regulation. This pathway involves ligand-induced internalization by endocytosis, followed by degradation in lysosomes, as seen in epidermal growth factor receptors (EGFR) and in platelet derived growth factor receptors (PDGFR) (40–42). In cancer cells, however, some of these deactivation pathways are compromised, leading to over-expression of RTKs (43, 44). It is highly likely that the regulation of Axl expression is disrupted in a similar manner in cancer, particularly in a hypoxic tumor microenvironment, and our group is focusing on this in our ongoing studies.

In conclusion, our data provides evidence for the role played by Axl and GAS6 in PCa tumorigenesis, and also offers insights into how GAS6/Axl signaling is regulated in PCa. While we still do not know how Axl expression is stabilized during hypoxia, a further understanding of this process may provide significant new therapeutic possibilities, especially when coupled with the knowledge that GAS6 signaling may regulate PCa dormancy in the marrow (26). In fact, it will be interesting to explore whether other RTK inhibitors which are being considered for as cancer therapeutic testing cancer will alter these

pathways, and whether they may be more effective with agents that prevent receptor stabilization (45, 46). Clearly, as evidence continues to mount that dormant microscopic tumors are prevalent in healthy individuals, understanding the molecular mechanisms that regulate dormancy will become increasingly important as our population ages.

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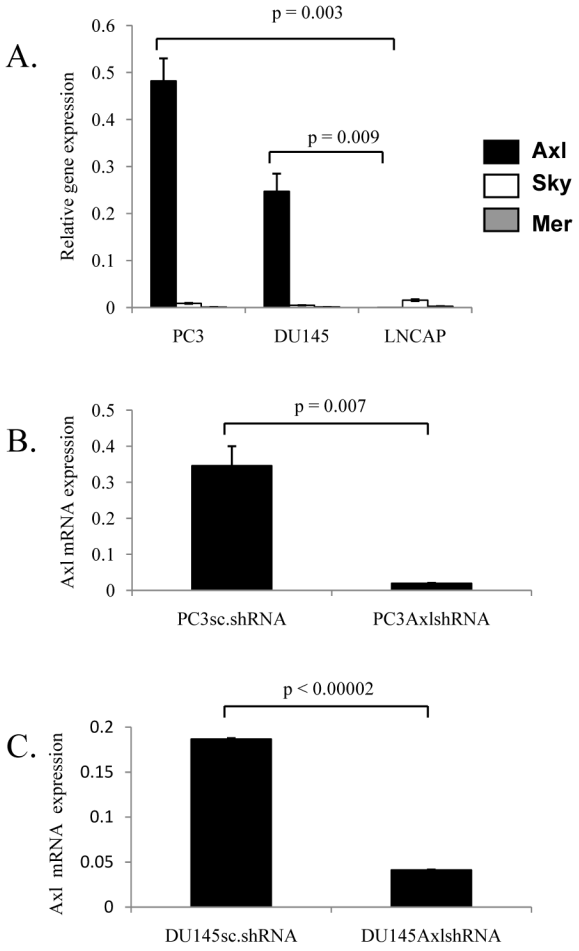


Figure 1. Axl is highly expressed in metastatic PCa cells

(A). Axl, Sky and Mer mRNA expression was analyzed by real time PCR in PC3, DU145, and LNCaP cells. (**B, C**). Following infection with lentivirus bearing Axl shRNA or scrambled shRNA (**sc.shRNA**), Axl mRNA levels were determined by real time PCR in PC3 and DU145 cells respectively. Gene expression shown is relative to β-actin levels.

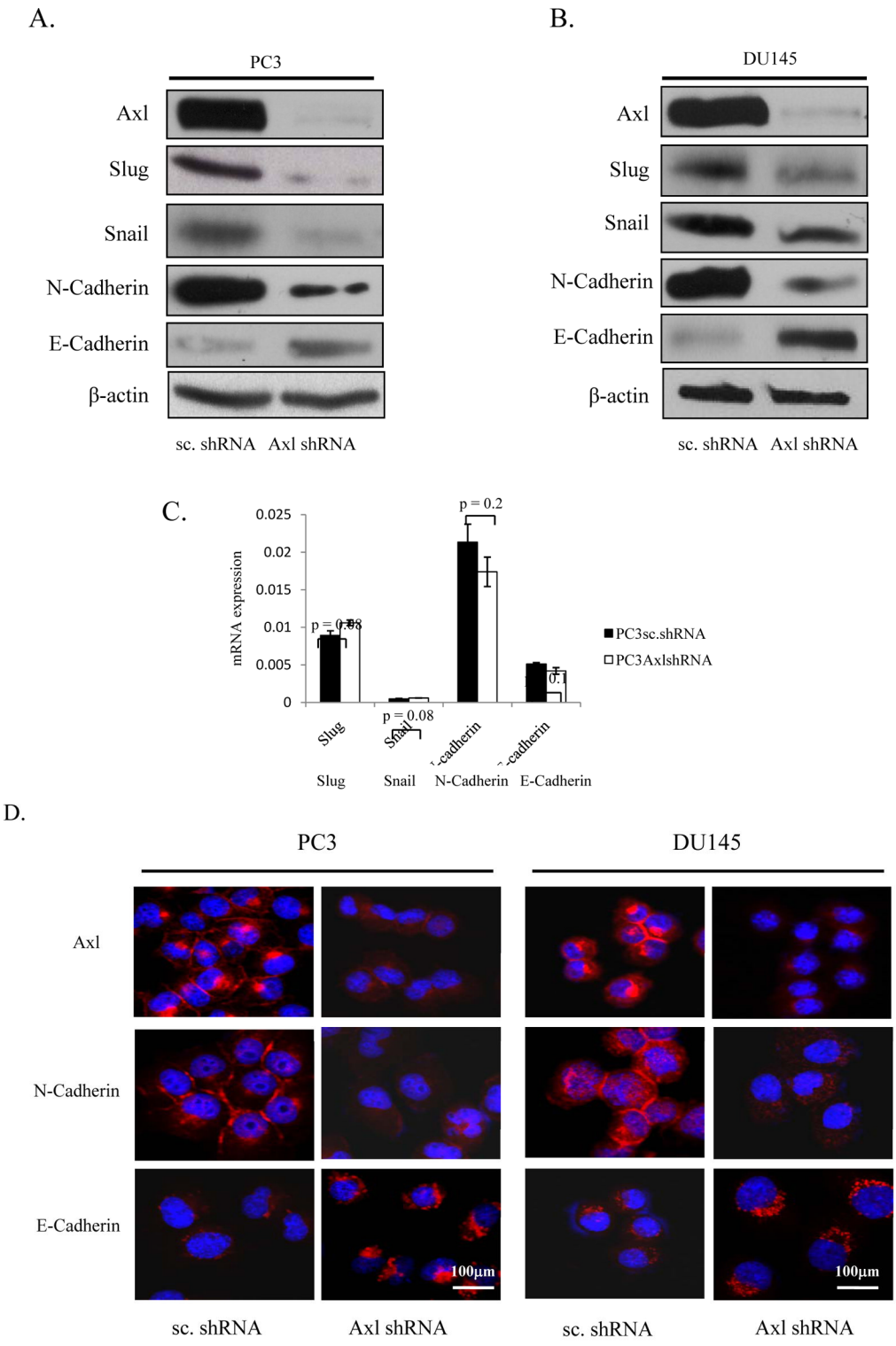


Figure 2. Axl regulates EMT in PCa cells

(**A, B**). Western blot analysis was used to determine protein levels of Axl, Slug, Snail, N-cadherin, E-cadherin, and β-actin in PC3 and DU145 with and without Axl shRNA. (**C**). mRNA levels of Axl, Slug, Snail, N-cadherin and E-cadherin relative to β-actin were

determined by real time PCR in PC3 cells with and without Axl shRNA. Values displayed here are fold differences in mRNA expression between control and Axl knockdown cells. sc.shRNA: scrambled shRNA. (**C**). Immnunohistochemical evaluation of Axl N-Caderhin and E-Cadherin expression following Axl shRNA knock down.

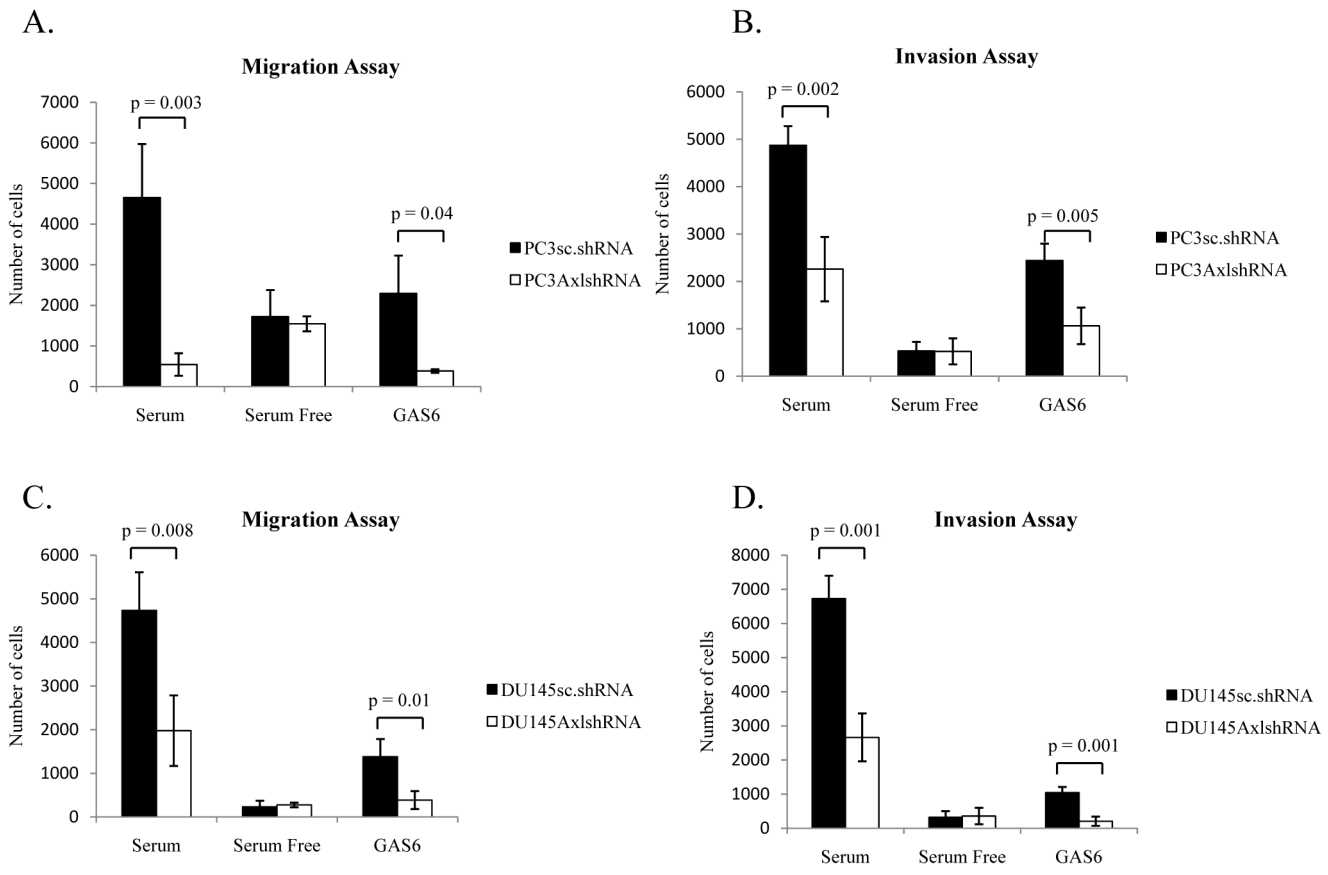


Figure 3. Knockdown of Axl in PCa cells leads to inhibition of migration and invasion *in vitro*

Following infection with lentiviruses bearing AxlshRNA or scrambled shRNA (**sc.shRNA**), migration(**A, C**) or invasion (**B, D**) assays were performed in Boyden chambers in either serum containing media, serum-free media, or serum-free media containing 100ng/ml GAS6 as a chemo attractant. (A, B) PC3. (C, D) DU145. Data are presented as mean ± standard deviation for triplicate determinations.

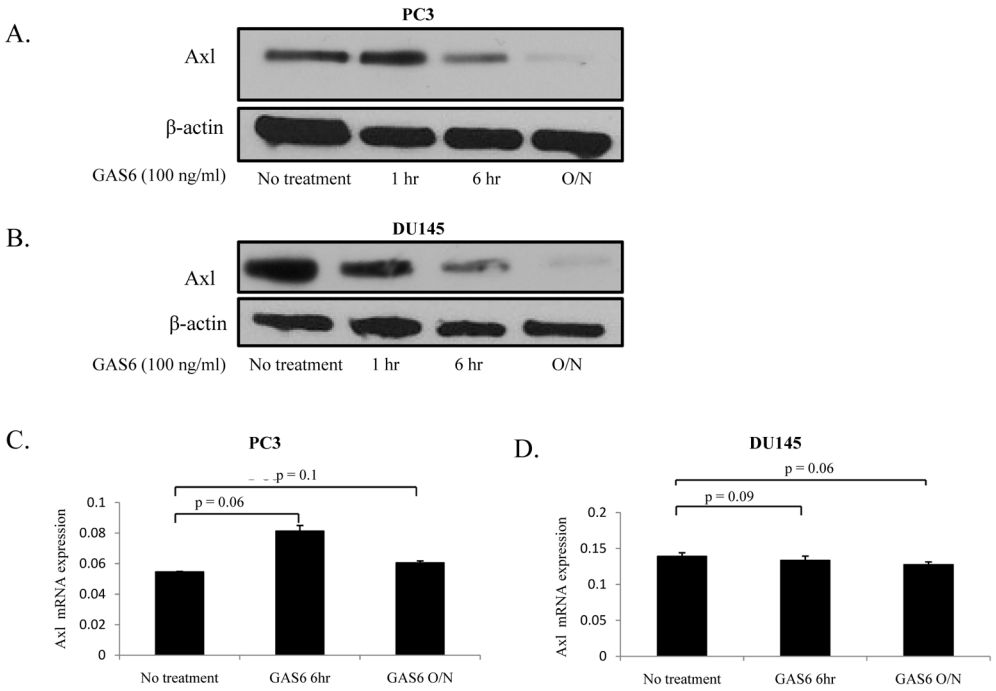


Figure 4. Exogenous treatment with GAS6 reduces Axl protein expression in PCa cells

Following overnight serum starvation, PC3 and DU145 cells were left untreated or treated with 100ng/ml of GAS6 for 6 hrs, or overnight (**O/N**). (**A, B**) Protein levels of Axl and β-actin (used as control) were determined by western blot analysis for PC3 and DU145. (**C, D**) mRNA levels for Axl were determined by real time PCR and normalized to β-actin levels.

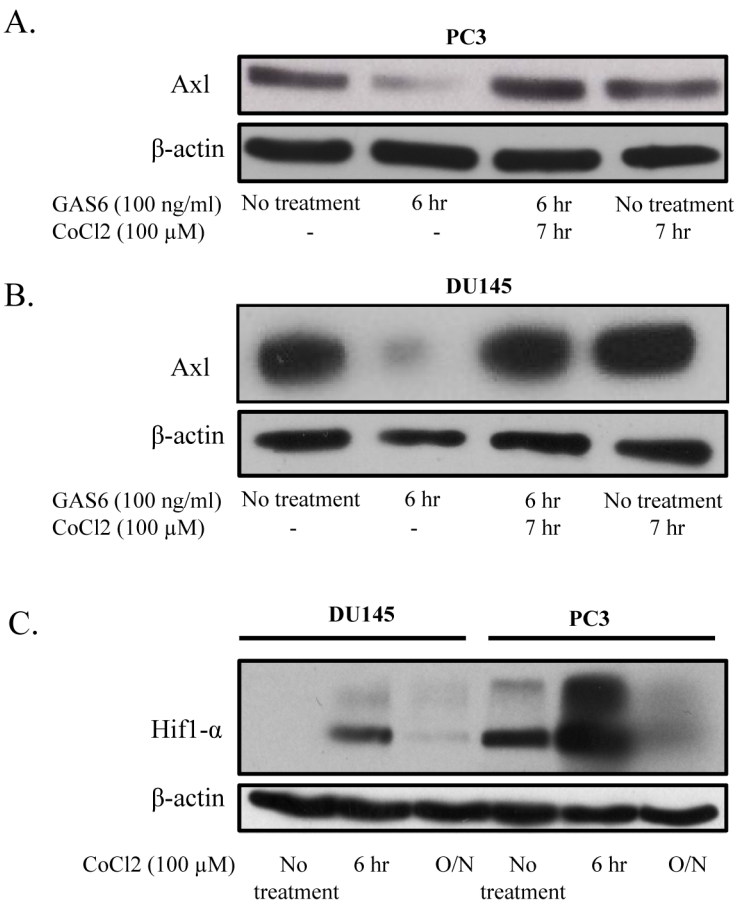
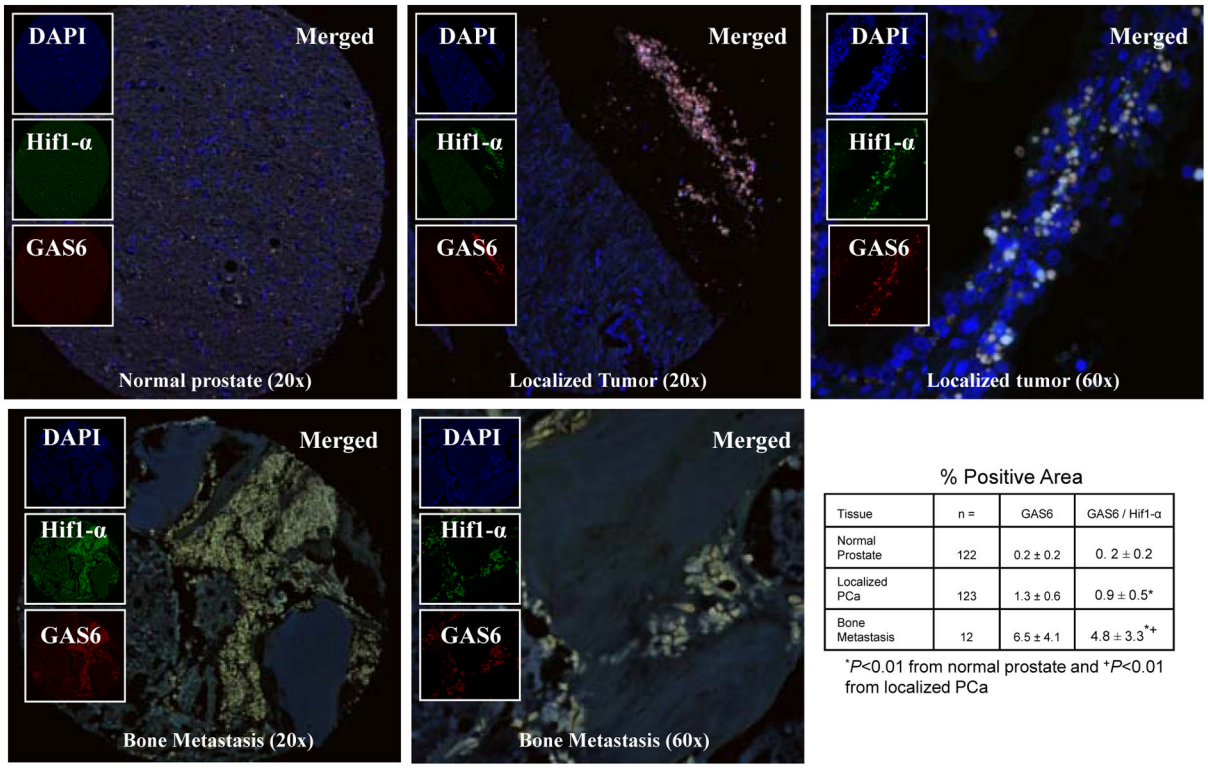


Figure 5. Hypoxia prevents GAS6 mediated degradation of Axl

Following overnight serum starvation, PC3 and DU145 cells were left untreated or treated with 100ng/ml of GAS6 for 6 hrs. One hour prior to GAS6 treatment some cells were also treated with 100 μM of CoCl2. (**A, B**) Protein levels of Axl and β-actin (used as control) were determined by western blot analysis (**C**) PC3 and DU145 cells were also treated with CoCl2 alone for 6 hrs and overnight (**O/N**) following which Hif1-α and β-actin protein levels were determined by western blot analysis.



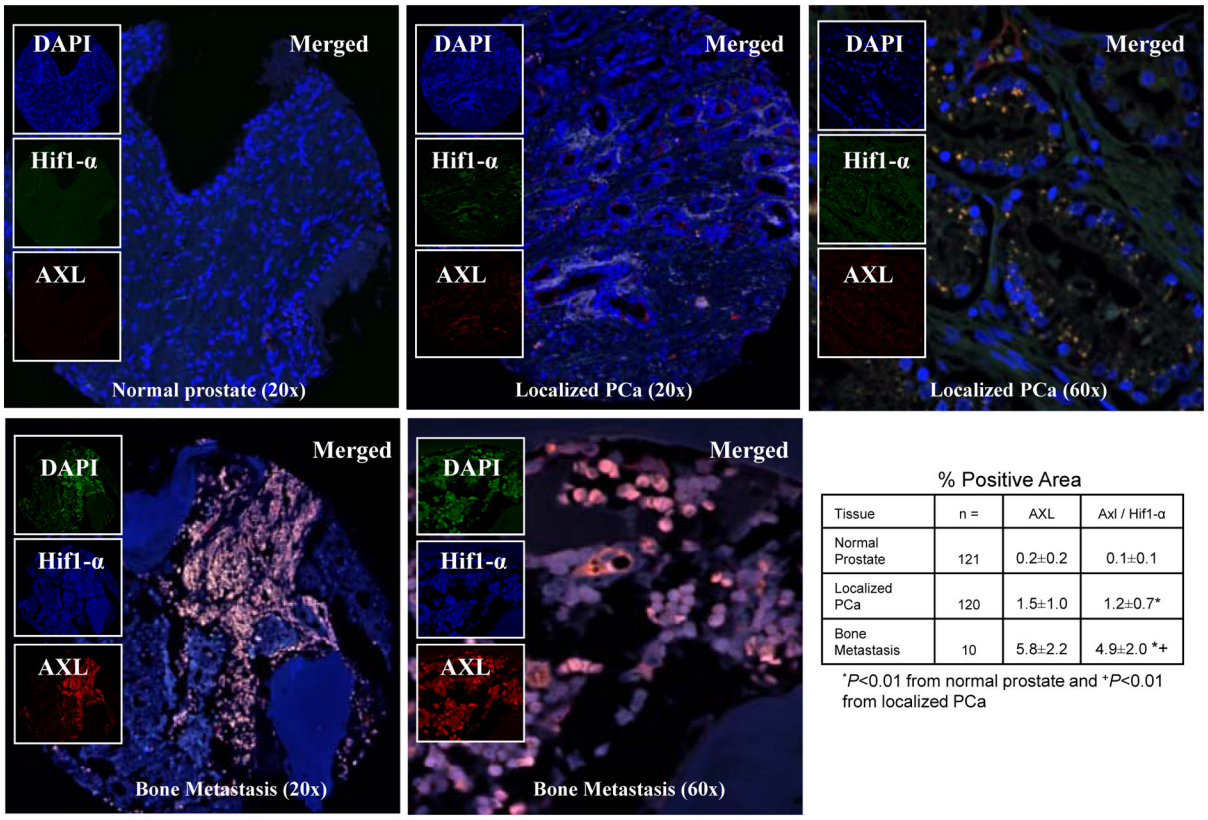
% Positive Area

Tissue	n =	GAS6	GAS6 / Hif1-α
Normal Prostate	122	0.2 ± 0.2	0. 2 ± 0.2
Localized PCa	123	1.3 ± 0.6	0.9 ± 0.5*
Bone Metastasis	12	6.5 ± 4.1	4.8 ± 3.3*+

*P<0.01 from normal prostate and +P<0.01 from localized PCa

Figure 6. GAS6 co-localizes with Hif1-α in both primary tumors and bone metastasis in human PCa

TMA samples were co-immunostained with DAPI, Hif1-α and GAS6. Representative micrographs showing normal prostate, localized PCa at 20x or 60x or bone metastasis at 20x or 60x. Quantification of the TMA images including the number of samples evaluated and % total area double positive, and was performed with Software NIS Elements BR3.2 64-bit (Nikon).



% Positive Area

Tissue	n =	AXL	Axl / Hif1-α
Normal Prostate	121	0.2±0.2	0.1±0.1
Localized PCa	120	1.5±1.0	1.2±0.7*
Bone Metastasis	10	5.8±2.2	4.9±2.0 *+

*P<0.01 from normal prostate and +P<0.01 from localized PCa

Figure 7. Axl co-localizes with Hif1-α in both primary tumors and bone metastasis in human prostate TMA samples

TMA samples were co-immunostained with DAPI, Hif1-α and Axl. Representative micrographs showing normal prostate at 20 x, localized prostate cancer at 20x or 60x and bone metastasis at 20x or 60x. Quantification of the TMA images including the number of samples evaluated and % total area double positive, and was performed with Software NIS Elements BR3.2 64-bit (Nikon).

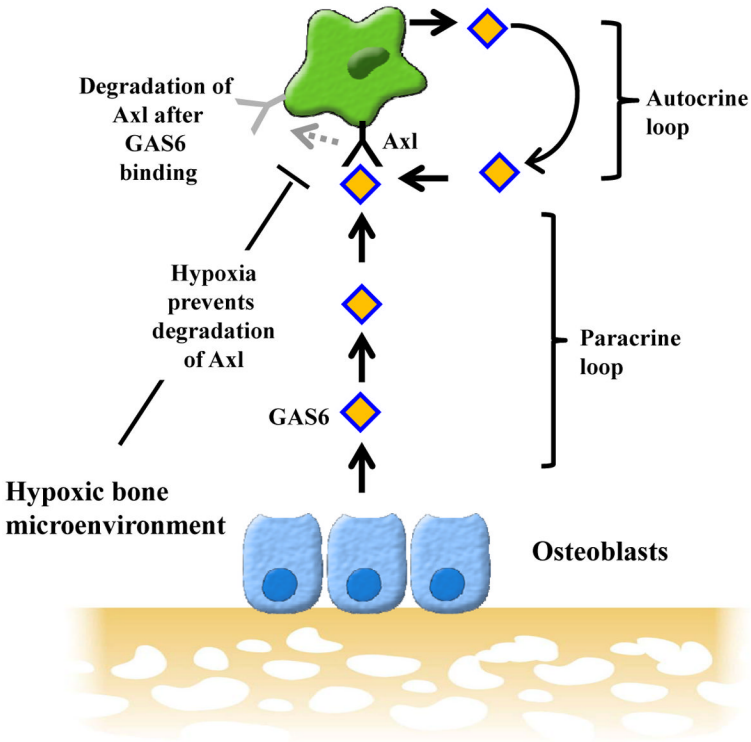


Figure 8. A model for Axl regulation in a hypoxic tumor microenvironment

In a bone marrow microenvironment GAS6 is either produced by osteoblasts (paracrine loop) or by the tumor cells (autocrine loop). The hypoxic microenvironment in the bone facilitates high expression of Axl by the tumor cells in spite of high GAS6 expression. This results in increased production of both the ligand and receptor which boosts GAS6/Axl signaling within the bone microenvironment, which enhances tumorigenesis.